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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No.	CRO01.NP001
First Inventor or Application Identifier	CROCE, Carlo M.
Title	NITRILASE HOMOLOGS
Express Mail Label No.	EE 388 355 653 US

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents.

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(preferred arrangement set forth below)
 - Descriptive title of the Invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
- ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets **6**]
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- ☐ Microfiche Computer Program (Appendix)
- Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
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- ☒ Assignment Papers (cover sheet & document(s))
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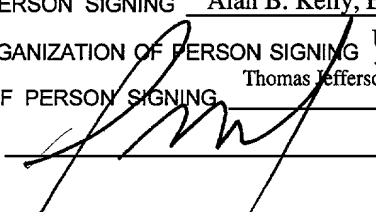
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<p>Applicant, Patentee, or Identifier: <u>CROCE, Carlo M.</u></p> <p>Application or Patent No.: <u>Not known</u></p> <p>Filed or Issued: <u>July 20, 1999</u></p> <p>Title: <u>NITRILASE HOMOLOGS</u></p> <p>I hereby state that I am an official empowered to act on behalf of the nonprofit organization identified below:</p> <p>NAME OF NONPROFIT ORGANIZATION <u>Thomas Jefferson University</u></p> <p>ADDRESS OF NONPROFIT ORGANIZATION <u>11th and Walnut Streets</u> <u>Philadelphia, PA 19107</u></p> <p>TYPE OF NONPROFIT ORGANIZATION:</p> <p><input checked="" type="checkbox"/> UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION</p> <p><input type="checkbox"/> TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3))</p> <p><input type="checkbox"/> NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA (NAME OF STATE _____) (CITATION OF STATUTE _____)</p> <p><input type="checkbox"/> WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA</p> <p><input type="checkbox"/> WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA (NAME OF STATE _____) (CITATION OF STATUTE _____)</p> <p>I hereby state that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in:</p> <p><input checked="" type="checkbox"/> the specification filed herewith with title as listed above.</p> <p><input type="checkbox"/> the application identified above.</p> <p><input type="checkbox"/> the patent identified above.</p> <p>I hereby state that rights under contract or law have been conveyed to and remain with the nonprofit organization regarding the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern, or organization having rights in the invention must file separate statements as to their status as small entities and that no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).</p> <p>Each person, concern, or organization having any rights in the invention is listed below:</p> <p><input checked="" type="checkbox"/> no such person, concern, or organization exists.</p> <p><input type="checkbox"/> each such person, concern, or organization is listed below.</p> <p>I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))</p> <p>NAME OF PERSON SIGNING <u>Alan B. Kelly, Esq.</u></p> <p>TITLE IN ORGANIZATION OF PERSON SIGNING <u>University Counsel</u></p> <p>ADDRESS OF PERSON SIGNING <u>Thomas Jefferson University, 1020 Walnut Street, Philadelphia, PA 19107</u></p> <p>SIGNATURE <u></u> DATE <u>7/20/99</u></p>	

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NITRILASE HOMOLOGS5 **FIELD OF THE INVENTION**

The present invention generally relates to the field of oncology and tumor suppressor genes, and more particularly to the structure and function of the *NIT1* gene, the structure of its encoded proteins, and the use of *NIT1* genes and the *NIT1* related genes and their encoded proteins and vectors containing the *NIT1* coding sequence as diagnostic and therapeutic reagents for the detection and treatment of cancer.

15 **BACKGROUND OF THE INVENTION***Introduction*

The present invention relates to nucleotide sequences of the *NIT1* gene and amino acid sequences of its encoded proteins, as well as derivatives and analogs thereof. Additionally, the present invention relates to the use of nucleotide sequences of *NIT1* genes and amino acid sequences of their encoded proteins and vectors containing the *NIT1* coding sequence, as well as derivatives and analogs thereof and antibodies thereto, as diagnostic and therapeutic reagents for the detection and treatment of cancer. The present invention also relates to therapeutic compositions comprising Nit1 proteins, derivatives or analogs thereof, antibodies thereto, nucleic acids encoding the Nit1 proteins, derivatives, or analogs, and *NIT1* antisense nucleic acids, and vectors containing the *NIT1* coding sequence.

30

Approaches to Elucidation and Characterization of *NIT1*

The tumor suppressor gene *FHIT* encompasses the common human
5 chromosomal fragile site at 3p14.2 and numerous cancer cell bi-allelic deletions.
To study Fhit function, *Fhit* genes in *D. melanogaster* and *C. elegans* were cloned
and characterized. The *Fhit* genes in both of these organisms code for fusion
proteins in which the Fhit domain is fused with a novel domain showing homology
to bacterial and plant nitrilases; the *D. melanogaster* fusion protein exhibited
10 diadenosine triphosphate (ApppA) hydrolase activity expected of an authentic Fhit
homolog.

In human and mouse, the nitrilase homologs and Fhit are encoded by two
different genes, *FHIT* and *NIT1*, localized on chromosomes 3 and 1 in human, and
14 and 1 in mouse, respectively. Human and murine *NIT1* genes were cloned and
15 characterized, their exon-intron structure, their patterns of expression, and their
alternative mRNA processing were determined.

The tissue specificity of expression of murine *FHIT* and *NIT1* genes was
nearly identical. Typically, fusion proteins with dual or triple enzymatic activities
have been found to carry out specific steps in a given biochemical or biosynthetic
20 pathway; Fhit and Nit1, as fusion proteins with dual or triple enzymatic activities,
likewise collaborate in a biochemical or cellular pathway in mammalian cells.

Importance of *FHIT*

25 The human *FHIT* gene at chromosome 3p14.2, spanning the constitutive
chromosomal fragile site FRA3B, is often altered in the most common forms of
human cancer and is a tumor suppressor gene. The human *FHIT* gene is greater
than one megabase in size encoding an mRNA of 1.1 kilobases and a protein of
147 amino acids.

30 The rearrangements most commonly seen are deletions within the gene.
These deletions, often occurring independently in both alleles and resulting in
inactivation, have been reported in tumor-derived cell lines and primary tumors of

lung, head and neck, stomach, colon, and other organs. In cell lines derived from several tumor types, DNA rearrangements in the *FHIT* locus correlated with RNA and/or Fhit protein alterations.

5 Because the inactivation of the *FHIT* gene by point mutations has not been demonstrated conclusively and because several reports have shown the amplification of aberrant-sized *FHIT* reverse transcription-PCR (RT-PCR) products from normal cell RNA, a number of investigators have suggested that the *FHIT* gene may not be a tumor suppressor gene. On the other hand it has been
10 reported that re-expression of Fhit in lung, stomach and kidney tumor cell lines lacking endogenous protein suppressed tumorigenicity *in vivo* in 4 out of 4 cancer cell lines. This suggests that *FHIT* is indeed a tumor suppressor gene. It is noted that a report has suggested that Fhit enzymatic activity is not required for its tumor suppressor function.

15 Fhit protein is a member of the histidine triad (HIT) superfamily of nucleotide binding proteins and is similar to the *Schizosaccharomyces pombe* diadenosine tetraphosphate (Ap₄A) hydrolase. Additionally it has been reported that, *in vitro*, Fhit has diadenosine triphosphate (ApppA) hydrolase enzymatic activity.

20 Neither the *in vivo* function of Fhit nor the mechanism of its tumor suppressor activity is known. Nonetheless, genetic, biochemical and crystallographic analysis suggest that the enzyme-substrate complex is the active form that signals for tumor suppression. One approach to investigate function is to investigate Fhit in model organisms such as *Drosophila melanogaster* and
25 *Caenorhabditis elegans*.

 The present invention involves the isolation and characterization of the *NIT1* gene in these organisms. Fhit occurs in a fusion protein, Nit-Fhit, in *D. melanogaster* and *C. elegans*, but *FHIT* and *NIT1* are separate genes in mammalian cells. The human and mouse *NIT1* genes are members of an
30 uncharacterized mammalian gene family with homology to bacterial and plant nitrilases, enzymes which cleave nitriles and organic amides to the corresponding carboxylic acids plus ammonia.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to purify a *NIT1* gene.

5 It is a further object of the present invention to purify a *NIT1* gene, wherein the purified gene is a human gene.

It is an object of the present invention to purify a *NIT1* gene, wherein the purified gene is a mammalian gene.

It is an object of the present invention to purify a Nit1 protein.

10 It is another object of the present invention to purify a Nit1 protein, wherein the purified protein is a human protein.

It is another object of the present invention to purify a Nit1 protein, wherein the purified protein is a mammalian protein.

Yet another aspect of the present invention is a purified protein encoded by
15 a nucleic acid having a nucleotide sequence consisting of the coding region of SEQ ID NO:1 (**Figure 6**).

Another aspect of the present invention is an antibody capable of binding a Nit1 protein.

It is another object of the present invention to isolate a nucleic acid of less
20 than 100 kb, comprising a nucleotide sequence encoding a Nit1 protein.

Another object of the present invention is a pharmaceutical composition comprising a therapeutically effective amount of a Nit1 protein; and a therapeutically acceptable carrier.

Another object of the present invention is a method of treating or
25 preventing a disease or disorder in a subject comprising administering to said subject a therapeutically effective amount of a molecule that inhibits Nit1 function.

Another aspect of the present invention is a method of treating or preventing a disease or disorder in a subject comprising administering to said subject a therapeutically effective amount of a molecule that enhances Nit1
30 function.

It is yet another aspect of the present invention to diagnose or screen for the presence of or a disposition for developing a disease in a subject, comprising

detecting one or more mutations in *NIT1* DNA, RNA or Nit1 protein derived from the subject in which the presence of said one or more mutations indicates the presence of the disease or disorder or a predisposition for developing the disease or disorder.

It is yet another aspect of the present invention to treat a disease or disorder with a vector containing the coding segment of the *NIT1* gene.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. A sequence comparison of human, murine, *D. melanogaster*, and *C. elegans* Nit1 and Fhit proteins. Identities are shown in black boxes, similarities are shown in shaded boxes. For human and mouse FHIT GenBank accession numbers are U46922 and AF047699, respectively.

Fig. 2. Northern blot analysis of expression of *NIT1* and *FHIT* mRNAs in murine and human tissues, as well as in *D. melanogaster*, and *C. elegans*. **(A)** Mouse multiple tissues Northern blot. Lanes 1-8: heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. (Top) *Fhit* probe; (Middle) *Nit1* probe; (Bottom) actin probe. **(B)** Human blot, *NIT1* probe. Lanes 1-8: heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. **(C)** Lanes 1 and 2: *D. melanogaster* adult, *D. melanogaster* embryo; *D. melanogaster* Nit-Fhit probe. Lane 3: *C. elegans* adult; *C. elegans* Nit-Fhit probe.

Fig. 3. Genomic organization of human and murine *NIT1* genes and *D. melanogaster* and *C. elegans* Nit-Fhit genes. **(A)** Exon-intron structure of the genes. **(B)** Alternative processing of human *NIT1* gene.

Fig. 4. Cleavage of ApppA by *D. melanogaster* Nit-Fhit. At indicated times of incubation, samples were spotted on TLC plates with appropriate nucleotide standards.

Fig. 5. Analysis of alternative transcripts of human *NIT1* by RT-PCR. RT-PCR of HeLa RNA was performed with primers in different exons. Lanes 1-6: exons 1 and 3 (transcript 2); exons 1C and 3 (transcript 5); exons 1A and 3

(transcripts 3, upper band and 4, lower band): exons 2 and 3 (transcripts 2-4); exons 1 and 1C (transcript 5); and exons 1 and 2 (transcript 2).

Fig. 6. Highly conserved sequence of human, murine, *D. melanogaster*,
5 and *C. elegans NIT1* gene. (SEQ ID NO:1).

DETAILED DESCRIPTION

10 Genomic and cDNA clones

One million plaques of a mouse genomic library (bacteriophage library from strain SVJ129, Stratagene, La Jolla, CA) and one hundred thousand plaques of a *D. melanogaster* genomic library were screened with corresponding cDNA
15 probes. Clones were purified and DNA was isolated. Sequencing was carried out using Perkin Elmer thermal cyclers and ABI 377 automated DNA sequencers. DNA pools from a human BAC library (Research Genetics, Huntsville, AL) were screened by PCR with *NIT1* primers (TCTGAAACTGCAGTCTGACCTCA (SEQ ID NO:2) and CAGGCACAGCTCCCCTCACTT (SEQ ID NO:3)) according to
20 the supplier's protocol. The DNA from the positive clone, 31K11, has been isolated using standard procedures and sequenced. Chromosomal localization of the human *NIT1* gene was determined using a radiation hybrid mapping panel (Research Genetics) according to the supplier's protocol and with the same primers as above. To map murine *Nit1* gene, Southern blot analysis of genomic
25 DNA from progeny of a (*AEJ/Gn-a bp^H/a bp^H* x *M. spretus*)F1 x *AEJ/Gn-a bp^h/a bp^h* backcross was performed using a full length murine *Nit1* cDNA probe. This probe detected a unique 2.0 kb *DraI* fragment in *AEJ* DNA and a unique 0.75 kb fragment in *M. spretus* DNA. Segregation of these fragments were followed in 180 N2 offspring of the backcross. Additional Mit markers (*D1Mit34*, *D1Mit35*,
30 and *D1Mit209*) were typed from DNA of 92 mice by using PCR consisting of an initial denaturation of 4 minutes at 94°C followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Linkage analysis was

performed using the computer program SPRETUS MADNESS: PART DEUX. Human and mouse *NIT1* expressed sequence tag (EST) clones were purchased from Research Genetics. The sequences of human and murine *NIT1* genes and
5 cDNAs and *D. melanogaster* and *C. elegans Nit-Fhit* cDNAs have been deposited in GenBank.

In situ hybridization

10 *D. melanogaster* polytene chromosome spreads were prepared from salivary glands of third-instar larvae as described. *NitFhit* DNA fragments were labeled with digoxigenin-11-dUTP using a random-primed DNA labeling kit (Boeringer Mannheim, Indianapolis, IN), and were used as probes for the chromosomal *in situ* hybridization. Hybridization was for 20 hours at 37°C in
15 hybridization buffer: 50% formamide, 2x standard saline citrate (SSC), 10% dextran sulfate, 400 mg/ml salmon sperm DNA. Antidigoxigenin-fluorescein antibodies (Boehringer Mannheim) were used for detection of hybridizing regions. DNA was counterstained with Hoechst 33258 (Sigma, St. Louis, MO). The slides were analyzed by fluorescence microscopy. For *in situ* hybridization, embryos
20 were fixed and processed as described previously, except that single-stranded RNA probes were used. Full length *NitFhit* cDNA was cloned into BluescriptII KS+ vector and used to synthesize antisense RNA probes with the Genius 4 kit (Boehringer Mannheim).

25 *RT-PCR, Northern and RACE analysis*

Human and mouse multiple tissue northern blots (Clontech, Palo Alto, CA) were hybridized with corresponding *NIT1* cDNA probes and washed using the supplier's protocol. For the HeLa cell line, total RNA was isolated from 1-5 x 10⁸
30 cells using Trizol reagent (Gibco BRL, Gaithersburg, MD). *D. melanogaster* PolyA+ RNA was purchased from Clontech. Three µg of polyA+ RNA or 15 µg of total RNA were electrophoresed in 0.8% agarose in a borate buffer containing

formaldehyde, transferred to HybondN+ membrane (Amersham, Arlington Heights, IL) using standard procedures and hybridized as described above. For RT-PCR, 200 ng of polyA+ RNA or 3 µg of total RNA were treated with DNaseI (amplification grade, Gibco BRL) following the manufacturer's protocol. DNase-treated RNA was used in reverse transcription (RT) reactions as follows: 10 nM each dNTP, 100 pmoles random hexamers (oligo (dT) priming was used in some cases), DNaseI treated RNA, and 200 units of murine leukemia virus (MuLV) reverse transcriptase (Gibco BRL), in total volume of 20 µl were incubated at 42°C for 1 hour followed by the addition of 10 µg RNase A and incubation at 37°C for 30 min. One µl of the reaction was used for each PCR reaction. PCR reactions were carried out under standard conditions using 10 pmoles of each gene-specific primer and 25-35 cycles of 95° 30", 55-60° 30", 72° 1'. Products were separated on 1.5% agarose gels and sometimes isolated and sequenced or cloned and sequenced. Oligo (dT)-primed double-stranded cDNA was synthesized by using procedures and reagents from the Marathon RACE cDNA amplification kit (Clontech); the cDNA was ligated to Marathon adapters (Clontech). 3' and 5' RACE products were generated by long PCR using gene-specific primers and the AP1 primer (Clontech). To increase the specificity of the procedure, the second PCR reaction was carried out by using nested gene-specific primers and the AP2 primer (Clontech). PCR reactions were performed according to the Marathon protocol using the Expand long template PCR system (Boehringer Mannheim) and 30 cycles of: 94° 30", 60° 30", 68° 4'. RACE products were electrophoresed, identified by hybridization and sequenced. Degenerate *FHIT* primers were: GTNGTNCCNGGNCA YGTNGT (SEQ ID NO:4) and ACRTGNACRTGYTTNACNGTYTGNGC (SEQ ID NO:5). *D. Melanogaster Fhit* RACE and RT-PCR primers were: GCGCCTTTGTGGCCTCGACTG (SEQ ID NO:6) and CGGTGGCGGAAGTTGTCTGGT (SEQ ID NO:7). *C. elegans Fhit* RACE and RT-PCR primers were: GTGGCGGCTGCTCAA AACTGG (SEQ ID NO:8) and TCGCGACGATGAACAAGTCGG (SEQ ID NO:9). Human *NIT1* RT-PCR primers were: GCCCTCCGGATCGGACCCT (SEQ ID NO:10) (exon 1); GACCTACTCCCTATCCCGTC (SEQ ID NO:11) (exon 1a);

GCTGCGAAGTGCACAGCTAAG (SEQ ID NO:12) and
 AACTGAAGCCTCTTTCCTCTGAC (SEQ ID NO:13) (exon 1c);
 TGGGCTTCATCACCAGGCCT (SEQ ID NO:14) and
 5 CTGGGCTGAGCACAAAGTACTG (SEQ ID NO:15) (exon 2);
 GCTTGTCTGGCGTCGATGTTA (SEQ ID NO:16) (exon 3).

Protein expression and enzymatic characterization

10 The *NIT-FHIT* cDNA was amplified with primers
 TGACGTCGACATATGTCAACTCTAGTTAATACCACG (SEQ ID NO:17) and
 TGGGTACCTCGACTAGCTTATGTCC (SEQ ID NO:18), digested with *Nde*I
 and *Kpn*I, and cloned into plasmid pSGA02 as a *Nde*I-*Kpn*I fragment.
Escherichia coli strain SG100 transformants were grown in Luria-Bertani with
 15 100 µg/ml of ampicillin and 15 µg/ml of chloramphenicol at 15°C. When the
 culture reached an optical density (600 nm) of 0.25, isopropyl β-D-thiogalactoside
 was added to a final concentration of 200 µM. NitFhit protein was purified from
 inclusion bodies as described. Briefly, the cell pellet from a 1-liter culture was
 resuspended in 50 ml of 20 mM Tris•HCl (pH 7.5), 20% sucrose, 1mM EDTA and
 20 repelleted. Outer cell walls were lysed by resuspension in ice-water. Spheroblasts
 were pelleted, resuspended in 140 mM NaCl, 2.7 mM KCl, 12 mM Na•P04 (pH
 7.3), 5mM EDTA, 500mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin and
 20 µg/ml of aprotinin, and sonicated. The resulting inclusion body preparation
 was washed and solubilized in 5 M guanidinium hydrochloride, 50mM Tris•HCl
 25 (pH 8.0), 5mM EDTA. Soluble NitFhit protein was added dropwise to 250ml of
 50mM Tris•HCl (pH 8.0), 1mM DTT, 20% glycerol at 40°C. After a 14 hour
 incubation, the 13-kg supernatant was concentrated 100-fold with a Centricon
 filter. A 1-liter culture yielded approximately 200 µg of partially purified, soluble
 NitFhit. ApppA hydrolase activity was assayed at 30°C in 20 µl of 50mM
 30 Na•HEPES pH 7.5, 10% glycerol, 0.5 mM MnCl₂, 4mM ApppA, 1 µM NitFhit.
 TLC plates were developed as described.

Cloning and characterization of *D. melanogaster* and *C. elegans*

Fhit homologs

5 To obtain *D. melanogaster Fhit* sequences, degenerate primers were designed in the conserved regions of exons 5 and 7 of human *FHIT*. RT-PCR experiments with these primers and *D. melanogaster* RNA resulted in an ~200 bp product, which when translated showed ~50% identity to human Fhit protein. This sequence was used to design specific *D. melanogaster Fhit* primers. 5' and 3'

10 RACE with these primers resulted in ~1.5 kb full length cDNA (including polyadenylation signal and Poly(A) tail) encoding a 460 amino acid protein with a 145 amino acid C-terminal part homologous to human Fhit (40% identity and 47% similarity) and a 315 amino acid N-terminal extension (**Fig. 1**). Northern analysis (**Fig. 2C**) showed a single band of ~1.5 kb in both embryo and adult *D.*

15 *melanogaster* confirming that the full length cDNA has been cloned.

 The 460 amino acid predicted protein sequence was used in a BLASTP search. Of the top 50 scoring alignments, 22 aligned with the 145 residue C-terminal segment (Fhit-related sequences) and 28 aligned with the 315 residue N-terminal segment. The 28 sequences aligning with the N-terminus were led by an

20 uncharacterized gene from chromosome X of *Saccharomyces cerevisiae* (P-value of 1.4×10^{-45}), followed by uncharacterized ORFs of many bacterial genomes and a series of enzymes from plants and bacteria that have been characterized as nitrilases and amidases. Thus, the 460 amino acid predicted protein contains an N-terminal nitrilase domain and a C-terminal Fhit domain and was designated

25 NitFhit.

 The *D. melanogaster Nit-Fhit* cDNA probe was used to screen a *D. melanogaster* lambda genomic library. Sequencing of positive clones revealed that the gene is intronless and, interestingly, the 1.5-kb *Nit-Fhit* gene is localized within the 1.6-kb intron 1 of the *D. melanogaster* homolog of the murine *glycerol*

30 *kinase (Gyk)* gene. The direction of transcription of the *Nit-Fhit* gene is opposite to that of the *Gyk* gene (**Fig. 3A**). It is not known if such localization affects transcriptional regulation of these two genes.

The cytological position of the *Nit-Fhit* gene was determined by *in situ* hybridization to salivary gland polytene chromosomes. These experiments showed that there is only one copy of the sequence which was localized to region 61A, at the tip of the left arm of chromosome 3. Digoxigenin-labeled RNA probes were hybridized to whole-mount embryos to determine the pattern of expression during development. *Nit-Fhit* RNA was uniformly expressed throughout the embryo suggesting that NitFhit protein could be important for most of the embryonic cells.

Because human Fhit protein and the *D. melanogaster* Fhit domain were only 40% identical, to show that the authentic *D. melanogaster* Fhit homolog was cloned, its enzymatic activity was tested. **Fig. 4** shows that recombinant *D. melanogaster* NitFhit is capable of cleaving ApppA to AMP and ADP and therefore possesses ApppA hydrolase activity.

C. elegans

Fhit genomic sequences were obtained from the Sanger database (contig Y56A3) by using BLAST searches. 5' and 3' RACE with *C. elegans* Fhit specific primers yielded a 1.4-kb cDNA (including polyadenylation signal and Poly(A) tail) coding for a 440 amino acid protein (**Fig. 1**). Northern analysis (**Fig. 2C**) showed a single band of a similar size in adult worms. Similarly to *D. melanogaster*, the *C. elegans* protein contained an N-terminal nitrilase domain and a C-terminal Fhit domain (**Fig. 1**) with 50% identity and 57% similarity to human Fhit. Comparison between *C. elegans* *Nit-Fhit* cDNA and genomic sequences from the Sanger database revealed that the *C. elegans* *Nit-Fhit* gene comprises 8 exons and is more than 6.5 kb in size (**Fig. 3A**); the nitrilase domain is encoded by exons 1-6, and the Fhit domain is encoded by exons 6-8. *D. melanogaster* and *C. elegans* NitFhit proteins are 50% identical and 59% similar and exhibit several conserved domains (**Fig. 1**).

Cloning and characterized of human and murine NIT cDNAs and genes

Because Fhit and nitrilase domains are part of the same polypeptides in *D.*
5 *melanogaster* and *C. elegans*, it is reasonable to suggest that they may be involved
in the same biochemical or cellular pathway(s) in these organisms. Because
nitrilase homologs are conserved in animals, the mammalian nitrilase homologs
were cloned as candidate Fhit-interacting proteins.

To obtain human and murine *NIT1* sequences, the *D. melanogaster* nitrilase
10 domain sequence was used in BLAST searches of the GenBank EST database.
Numerous partially sequenced human and murine *NIT1* ESTs were found. All
mouse *Nit1* ESTs were identical, as were all human *NIT1* ESTs, suggesting the
presence of a single *NIT1* gene in mouse and human. To obtain the full-length
human and mouse cDNAs, several human and mouse ESTs and human 5' and 3'
15 RACE products were completely sequenced. This resulted in the isolation of a
~1.4-kb full-length human sequence encoding 327 amino acids and a ~1.4-kb
mouse full-length sequence coding for 323 amino acids (**Fig. 1**), although several
alternatively spliced products were detected in both cases (see below and **Fig. 3B**).
Both cDNAs are polyadenylated, but lack polyadenylation signals, although AT-
20 rich regions are present at the very 3' end of each cDNA. Mouse and human *Nit1*
amino acid sequences were 90% identical; the human *Nit1* amino acid sequence
was 58% similar and 50% identical to the *C. elegans* nitrilase domain and 63%
similar and 53% identical to the *D. melanogaster* nitrilase domain (**Fig. 1**).

Murine lambda and human BAC genomic libraries were screened with the
25 corresponding *NIT1* cDNA probes, yielding one mouse lambda clone and one
human BAC clone containing the *NIT1* genes. The human and murine *NIT1*
genomic regions were sequenced and compared to the corresponding cDNA
sequences. The genomic structure of human and mouse *NIT1* genes is shown in
Fig. 3A. Both genes are small: the human gene is ~3.2 kb in size and contains 7
30 exons; the murine gene is ~3.6 kb in size and contains 8 exons. Southern analysis
confirmed that both human and mouse genomes harbor a single *NIT1* gene.

A radiation hybrid mapping panel (GeneBridge 4) was used to determine the chromosomal localization of the human *NIT1* gene. By analysis of PCR data at the Whitehead/MIT database (<http://www-genome.wi.mit.edu>), the *NIT1* gene was localized 6.94 cR from the marker CHLC.GATA43A04, which is located at 1q21-1q22.

A full length murine *Nit1* cDNA probe was used to determine the chromosomal location of the murine gene by linkage analysis. Interspecific backcross analysis of 180 N₂ mice demonstrated that the *Nit1* locus cosegregated with several previously mapped loci on distal mouse chromosome 1. The region to which *Nit1* maps was further defined by PCR of genomic DNA from 92 N₂ mice using the markers *D1Mit34*, *D1Mit35* and *D1Mit209* (Research Genetics). The following order of the genes typed in the cross and the ratio of recombinants to N₂ mice was obtained: centromere - *D1Mit34* - 7/78 - *D1Mit35* - 8/90 - *Nit1* - 11/91 - *D1Mit209* - telomere. The genetic distances given in centiMorgans (\pm S.E.) are as follows: centromere - *D1Mit209* - 9.0 ± 3.2 - *D1Mit35* - 8.9 ± 3.0 - *Nit1* - 12.1 ± 3.4 - *D1Mit209* - telomere. This region of mouse chromosome 1 (1q21 – 1q23) is syntenic to human chromosome 1q and is consistent with the localization of the human ortholog of *Nit1*.

Expression and alternative splicing of human and murine *Nit1* genes

For the human gene, Northern analysis revealed two major transcripts of ~1.4 kb and ~2.4 kb in all adult tissues and tumor cell lines tested. A third band of ~1.2 kb was observed in adult muscle and heart (**Fig. 2B**). The longest cDNA (~1.4 kb) corresponds to the ~1.4-kb transcript observed on Northern blots. The 1.2-kb band corresponds to transcript 1 on **Fig. 3B** (see below). It is not known if the ~2.4-kb RNA represents an additional transcript or an incompletely processed mRNA. No significant variation in human *NIT1* mRNA levels was observed in different tissues (**Fig. 2B**). On the contrary, different mouse tissues showed different levels of expression of *Nit1* mRNA (**Fig. 2A**). The highest levels of *Nit1* mRNA were observed in mouse liver and kidney (**Fig. 2A, Middle**, lanes 5 and 7).

Interestingly, the pattern of *Nit1* expression was almost identical to the pattern of the expression of *Fhit* (**Fig. 2A**, *Top* and *Middle*), supporting the hypothesis that the proteins may act in concert or participate in the same pathway.

5 Analysis of mouse *Nit1* ESTs revealed that some transcripts lack exon 2 and encode a 323 amino acid protein. An alternative transcript containing exon 2 encodes a shorter, 290 amino acid protein starting with the methionine 34 (**Fig. 1**).

10 Analysis of human ESTs and 5' RACE products from HeLa and testis also suggested alternative processing. To investigate this, a series of RT-PCR experiments was carried out. **Fig. 5** shows the results obtained from HeLa RNA (similar results were obtained using RNAs from the MDA-MB-436 breast cancer cell line and adult liver). The alternatively spliced transcripts are shown on **Fig. 3B**. Transcript 1, lacking exon 2, was represented by several ESTs in the Genbank EST database. This transcript probably corresponds to the ~1.2-kb transcript
15 observed on Northern blots in adult muscle and heart. Transcript 2 encoding the 327 amino acid Nit1 protein (**Fig. 1**) is a major transcript of human *NIT1* at least in the cell lines tested. This transcript lacks exons 1a and 1b. Transcript 3 has exon 1a and 1b; transcript 4 has exon 1a but lacks exon 1b (**Fig. 3B**). It is not known if transcript 5 (lacking exon 2) starts from exon 1 or 1c.

20 The alternative initiating methionines of different transcripts are shown on **Fig. 3B**. Data suggest that at least in COS-7 cells transfected with a construct containing transcript 2, the methionine in exon 3 (shown in transcripts 1 and 3, **Fig. 3B**) initiates more efficiently than the methionine in exon 2 (**Fig. 3B**, transcript 2).

25

Discussion

Although the frequent loss of *Fhit* expression in several common human cancers is well documented, and results supporting its tumor suppressor activity
30 have been reported, the role of *Fhit* in normal and tumor cell biology and its mechanism of its action *in vivo* are unknown. The Ap₃A hydrolytic activity of *Fhit* seems not to be required for its tumor suppressor function, and it has been

suggested that the enzyme-subtract complex is the active form of Fhit. To facilitate an investigation of Fhit function, a model organisms approach was initiated by cloning and characterization of *D. melanogaster* and *C. elegans* Fhit genes.

Surprisingly, in flies and worms, Fhit is expressed as a fusion protein with the Fhit domain fused into a "Nit" domain showing homology to plant and bacterial nitrilases. Human and murine *NIT1* genes were further isolated. Nit and Fhit are expressed as separate proteins in mammals but, at the mRNA level, are coordinately expressed in mouse tissues.

In several eukaryotic biosynthetic pathways multiple steps are catalyzed by multifunctional proteins containing two or more enzymatic domains. The same steps in prokaryotes frequently are carried out by monoenzymatic proteins that are homologs of each domain of the corresponding eukaryotic protein. For example, Gars, Gart and Airs are domains of the same protein in *D. melanogaster* and mammals. These domains catalyze different steps in *de novo* synthesis of purines. In yeast, Gart homolog (Ade8) is a separate protein and Gars and Airs homologs (Ade5 and Ade7) are domains of a bienzymatic protein; in bacteria, all three homologs (PurM, PurN and PurD) are separate proteins. *De novo* pyrimidine biosynthesis illustrates a similar case. Recently, a fusion protein of a lipoyxygenase and catalase, both participating in the metabolism of fatty acids, has been identified in corals. In all of these examples, if domains of a multienzymatic protein in some organisms are expressed as individual proteins in other organisms, the individual proteins participate in the same pathways. This observation and the fact that Fhit and Nit1 exhibit almost identical expression patterns in murine tissues suggest that Fhit and Nit1 participate in the same cellular pathway in mammalian cells.

WHAT IS CLAIMED IS:

1. A purified *NIT1* gene.
2. The gene of claim 1 which is a human gene.
3. The gene of claim 1 which is a mammalian gene.
4. A purified Nit1 protein.
5. The protein of claim 4 which is a human protein.
6. A purified protein encoded by a nucleic acid having a nucleotide sequence consisting of the coding region of SEQ ID NO:1.
7. An antibody which is capable of binding a Nit1 protein.
8. The antibody of claim 7 which is monoclonal.
9. A molecule comprising a fragment of the antibody of claim 7, which fragment is capable of binding a Nit1 protein.
10. An isolated nucleic acid of less than 100 kb, comprising a nucleotide sequence encoding a Nit1 protein.
11. The nucleic acid of claim 10 in which the Nit1 protein is a human Nit1 protein.
12. A pharmaceutical composition comprising a therapeutically effective amount of a Nit1 protein; and a therapeutically acceptable carrier.

13. A method of treating or preventing a disease or disorder in a subject comprising administering to said subject a therapeutically effective amount of a molecule that inhibits Nit1 function.

5

14. A method of treating or preventing a disease or disorder in a subject comprising administering to said subject a therapeutically effective amount of a molecule that enhances Nit1 function.

10

15. A method of diagnosing or screening for the presence of or a predisposition for developing a disease or disorder in a subject comprising detecting one or more mutations in *NIT1* DNA, RNA or Nit1 protein derived from the subject in which the presence of said one or more mutations indicates the presence of the disease or disorder or a predisposition for developing the disease or disorder.

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16. A method of treating or preventing a disease or disorder in a subject by using a vector containing the *NIT1* gene coding sequence.

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ABSTRACT

The present invention relates to nucleotide sequences of the *NIT1* gene and
5 amino acid sequences of its encoded proteins, as well as derivatives and analogs
thereof. Additionally, the present invention relates to the use of nucleotide
sequences of *NIT1* genes and amino acid sequences of their encoded proteins, as
well as derivatives and analogs thereof and antibodies thereto, as diagnostic and
therapeutic reagents for the detection and treatment of cancer. The present
10 invention also relates to therapeutic compositions comprising Nit1 proteins,
derivatives or analogs thereof, antibodies thereto, nucleic acids encoding the Nit1
proteins, derivatives, or analogs and *NIT1* antisense nucleic acids, and vectors
containing the *NIT1* coding sequence.

```

hu_Fhit 1 ~~~~~
mu_Fhit 1 ~~~~~
hu_Nit1 1 MLGFTITPPPHRFLSLLCPGLRIPQLSVLCAQPPRAMAISESSCELPVAVCCVTSSTPPKQONEKTCABTVREHARLCAGCLAFLEPAFDF
mu_Nit1 1 MLGFTITPPPHQ...LLCTGYRLLRTPVLCTQPPRTMS.SSTWELPLVAVCCVTSSTPPKQONEKTCABTVREHARLCAGCLAFLEPAFDF
dr_Nitfhit 1 ~~~~~
ce_Nitfhit 1 ~~~~~MLSTVFRRMATGRHFVAVCCVTSSTPPKQONEKTCABTVREHARLCAGCLAFLEPAFDF

hu_Fhit 1 ~~~~~
mu_Fhit 1 ~~~~~
hu_Nit1 91 HADPAETGHLSEPTGCKATEETOLARECCGLHSLGCPHERCQDWEQTKIYNCHVLNLSKGAVVVRYRRTHLCQVEIFGQGPMSCSNS
mu_Nit1 87 HARNPAETLLSEPTGCKATEETOLARECCGLHSLGCPHERCQDWEQTKIYNCHVLNLSKGAVVVRYRRTHLCQVEIFGQGPMSCSNS
dr_Nitfhit 38 YGESRTCTIELSEGDDGETMAQVRELAKCNKIWISLGCVHER..NDQKI...HNAHVLNLSKGAVVVRYRRTHLCQVEIFGQGPMSCSNS
ce_Nitfhit 59 HCLNKNKQZDDAMATDCYMEKVRELARKNNIWLISLGCLEHKE...DPSDAAAPHTHLLIDSCVTRRKHNLHFDLEIFGKVRLEMSSEF

hu_Fhit 1 ~~~~~
mu_Fhit 1 ~~~~~
hu_Nit1 181 HMGCPSEHSCVCTEACGLCPAVCYDMFFPELSDAHAQAAGETLTPSAFGSITGPAHWEVLLRARAIETQCYVVAAGACGRHHPKRSYSG
mu_Nit1 177 HPGGGLSEPVVTEBAGKVCARQYDAPPELSEKPAQAAGETLTPSAFGSITGPAHWEVLLRARAIETQCYVVAAGACGRHHPKRSYSG
dr_Nitfhit 122 VTGVCLEPEVSTPVGQICLCICYDASREPAVLARKLCANTLTPSAFTYATGCAHWEVLLRARAIETQCYVVAAGACGRHHPKRSYSG
ce_Nitfhit 147 HKAETEMIPPVDTETGRLCMSICYDVRFFPELSYNNARKGAQCLSTPSAFTLNGCAHWEVLLRARAIETQCYVVAAGACGRHHPKRSYSG

hu_Fhit 1 ~~~~~
mu_Fhit 1 ~~~~~
hu_Nit1 271 HSMVVDPEWGTAVARCSBGP.GYGLARRIDINYPQRLRHLPVFCRRRPDLYGWLGHPTG~~~~~MSFREGQHLLKPSVVVLEKTELSEFALVN
mu_Nit1 267 HSMVVDPEWGTAVARCSBGP.GYGLARRIDINYPQRLRHLPVFCRRRPDLYGWLGHPTG~~~~~MSFREGQHLLKPSVVVLEKTELSEFALVN
dr_Nitfhit 212 HSMVVDPEWGTAVARCSBGP.QELDIGTAEVDLSVLOSHTQMPFCRRRPDLYGWLGHPTG~~~~~MSFREGQHLLKPSVVVLEKTELSEFALVN
ce_Nitfhit 237 HSMVVDPEWGTAVARCSBGP.VDMGFLPEHDLSEYDTRERMPVFSRRRSDLYTL.HINEKSSE.TGGLKPARFNPADHIFYSQPHSFFVFN

hu_Fhit 28 RKPVVFGHVLVCELPVERFRDHPDEVDADLEQVTRQVGYVVEKHFGTSLEFPMODCPAGQTVRHVVVHVPBRACGDFHNDSTIYEEF
mu_Fhit 28 RKPVVFGHVLVCELPVERFRDHPDEVDADLEQVTRQVGYVVEKHFGTSLEFPMODCPAGQTVRHVVVHVPBRACGDFHNDSTIYEEF
hu_Nit1 328 RKPVVFGHVLVCELPVERFRDHPDEVDADLEQVTRQVGYVVEKHFGTSLEFPMODCPAGQTVRHVVVHVPBRACGDFHNDSTIYEEF
mu_Nit1 324 RKPVVFGHVLVCELPVERFRDHPDEVDADLEQVTRQVGYVVEKHFGTSLEFPMODCPAGQTVRHVVVHVPBRACGDFHNDSTIYEEF
dr_Nitfhit 302 LKCVVFGHVLVSTRRVVPRLCGLDCAEMADMETTVCLVORTLEKRYKTSAPVYVODGCAQAGQTVRHVVHHPBRACGDFHNDSTIYEEF
ce_Nitfhit 324 LKCVVFGHVLVSTRRVVPRLCGLDCAEMADMETTVCLVORTLEKRYKTSAPVYVODGCAQAGQTVRHVVHHPBRACGDFHNDSTIYEEF

hu_Fhit 118 QKHDK...DFHAWRSEEMAAZAAARVYFQA-
mu_Fhit 118 QKHDK...DFHAWRSEEMAAZAAARVYFQA-
hu_Nit1 328 QKHDK...DFHAWRSEEMAAZAAARVYFQA-
mu_Nit1 324 QKHDK...DFHAWRSEEMAAZAAARVYFQA-
dr_Nitfhit 392 ..DEKARPKPRTIEERIEENQIYRKFLTDIS-
ce_Nitfhit 413 ASHDKPEPRRRGRNEQMADEAVVYRNFM-----

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Fig. 1

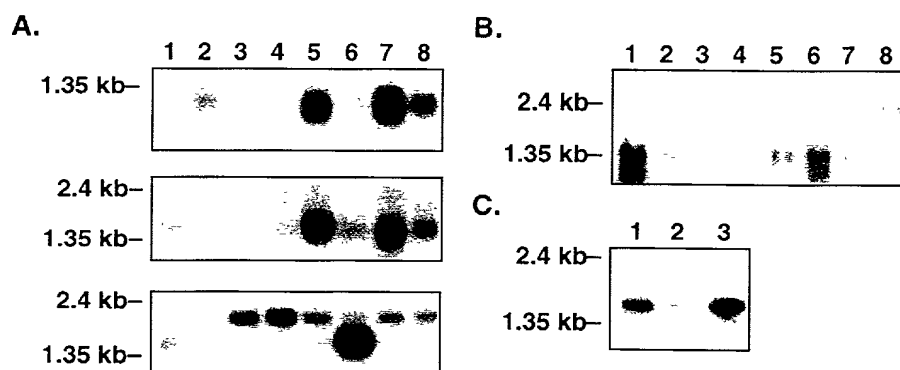


Fig. 2

intron: 1a; 198bp 1b; 243bp 2; 252bp 3; 120bp 4; 206bp 5; 154bp 6; 266bp

exons: 1; 1a, 1b; >115bp 1c; >110bp 2; 96bp 3; 255bp 4; 104bp 5; 134bp 6; 126bp 7; 568bp

Murine *Nit1*

intron: 1; 256bp 2; 190bp 3; 374bp 4; 132bp 5; 243bp 6; 130bp 7; 640bp

exons: 1; >65bp 2; 124bp 3; 87bp 4; 252bp 5; 97bp 6; 134bp 7; 126bp 8; 553bp

C. elegans NitFhit

intron: 1; >1.6 kb 2; 158bp 3; 46bp 4; 2046bp 5; >1.5 kb 6; 63bp 7; 52bp

exons: 1; >50bp 2; 96bp 3; 157bp 4; 156bp 5; 310bp 6; 225bp 7; 176bp 8; 202bp

D. melanogaster NitFhit

gyk exon 2 1.5 kb intronless gyk exon 1



0' 30' 60' 2h 4h 8h 15h



ApppA

AMP

ADP

Fig. 4

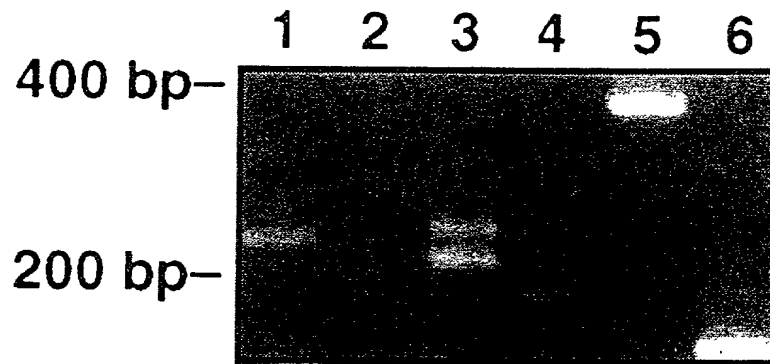


Fig. 5

NTID
[Strand]

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1  GCCCACTCGC TGCGGCCTINT CTGGCTCCAG ACCGCCCTCC GGATCGGACC CTGCGAATGG
   P L A A A ? L A P D R P P D R T L R M V
61  TTTTGGCTAT ATCTTCATGT AGGACCTACT CCCTATCCCG TCGGCCGCGG CTGGGCTTCA
   L A I S S C R T Y S L S R R P R L G F I
121 TCACCAGGCC TCCTCACAGA TTCTGTGCC TTCTGTGTCC TGGACTCCGG ATACCTCAAC
   T R P P H R F L S L L C P G L R I P Q L
181 TCTCAGTACT TTGTGCTCAG CCCAGGCCCA GAGCCATGGC TATCTCCTCT TCCTCCTGCG
   S V L C A Q P R P R A M A I S S S S C E
241 AACTGCCCCCT GGTGGCTGTG TGCCAGGTAA CATCGACGCC AGACAAGCAA CAGAACTTTA
   L P L V A V C Q V T S T P D K Q Q N F K
301 AAACATGTGC TGAGCTGGTT CGAGAGGCTG CCAGACTGGG TGCCTGCGCTG GCTTTCTGTC
   T C A E L V R E A A R L G A C L A F L P
361 CTGAGGCATT TGACTTCATT GCACGGGACC CTGCAGAGAC GCTACACCTG TCTGAACCAC
   E A F D F I A R D P A E T L H L S E P L
421 TGGGTGGGAA ACTTTTGGAA GAATACACCC AGCTTGCCAG GGAATGTGGA CTCTGGCTGT
   G G K L L E E Y T Q L A R E C G L W L S
481 CCTTGGGTGG TTTCCATGAG CGTGGCCAAG ACTGGGAGCA GACTCAGAAA ATCTACAATT
   L G G F H E R G Q D W E Q T Q K I Y N C
541 GTCACGTGCT GCTGAACAGC AAAGGGGCGAG TAGTGGCCAC TTACAGGAAG ACACACTTGT
   H V L L N S K G A V V A T Y R K T H L C
601 GTGACGTAGA GATTCCAGGG CAGGGGCCTA TGTGTGAAAG CAACTCTACC ATGCCTGGGC
   D V E I P G Q G P M C E S N S T M P G P
661 CCAGTCTTGA GTCACCTGTC AGCACACCAG CAGGCAAGAT TGGTCTAGCT GTCTGCTATG
   S L E S P V S T P A G K I G L A V C Y D
721 ACATGCGGTT CCCTGAATC TCTCTGGCAT TGGCTCAAGC TGGAGCAGAG ATACTTACCT
   M R F P E L S L A L A Q A G A E I L T Y
781 ATCCTTCAGC TTTTGGATCC ATTAACAGCC CAGCCACTG GGAGGTGTTG CTGCGGGCCC
   P S A F G S I / T G P A H W E V L L R A R
841 GTGCTATCGA AACCCAGTGC TATGTAGTGG CAGCAGCACA GTGTGGACGC CACCATGAGA
   A I E T Q C Y V V A A A Q C G R H H E K
901 AGAGAGCAAG TTATGGCCAC AGCATGGTGG TAGACCCCTG GGAACAGTG GTGGCCCGCT
   R A S Y G H S M V V D P W G T V V A R C
961 GCTCTGAGGG GCCAGGCCTC TGCCTTGCCC GAATAGACCT CAACTATCTG CGACAGTTGC
   S E G P G L C L A R I D L N Y L R Q L R
1021 GCCGACACCT GCCTGTGTTT CAGCACCGCA GGCCTGACCT CTATGGCAAT CTGGGTCACC
   R H L P V F Q H R R P D L Y G N L G H P
1081 CACTGTCTTA AGACTTGACT TCTGTGAGTT TAGACCTGCC CTTCCCACCC CCACCCTGCC
   L S . D L T S V S L D L P L P P P P C H
1141 ACTATGAGCT AGTGCTCATG TGACTTGGAG GCAGGATCCA GCCACAGCTC CCCTCACTTG
   Y E L V L M . L G G R I Q A Q L P S L G
1201 GAGAACCTTG ACTCTCTTGA TGGAACACAG ATGGGCTGCT TGGGAAAGAA ACTTTCACCT
   E P . L S . W N T D G L L G K E T F T .
1261 GAGCTTCACC TGAGGTCAGA CTGCAGTTTC AGAAAGGTGG AATTTTATAT AGTCATTGTT
   A S P E V R L Q F Q K G G I L Y S H C L
1321 TATTTTCATG AACTGAAGT TCTGCTGAGG GCTGAGCAGC ACTGGCATTG AAAAATATAA
   F H G N . S S A E G . A A L A L K N I I
1381 TAATCATAAA GTCAAAAAAA AAAAAA AAAA
   I I K S K K K K K K K

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Fig. 6

Please type a plus sign (+) inside this box → **+**

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	First Named Inventor	CROCE, Carlo M.
	COMPLETE IF KNOWN	
	Application Number	/
	Filing Date	July 20, 1999
	Group Art Unit	
Examiner Name		

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NITRILASE HOMOLOGS

the specification of which (Title of the Invention)
☒ is attached hereto
OR
☐ was filed on (MM/DD/YYYY) [] as United States Application Number or PCT International Application Number [] and was amended on (MM/DD/YYYY) [] (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

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Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
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			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

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60/093,350	07/20/1998	

[Page 1 of 2]

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U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

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☒ Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number
Clifford Kent Weber, Esq.	42,215		

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

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Name of Sole or First Inventor:		<input type="checkbox"/> A petition has been filed for this unsigned inventor			
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Post Office Address					
City	Philadelphia	State	PA	ZIP	19103
				Country	USA

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